REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
2012	Open Literature	

4. TITLE AND SUBTITLE
Tertiary Oximes on Brain Acetylcholinesterase and Central Excitatory Effects of Nerve Agents

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Shih, T-M, Koenig, J.A., McDonough, JH

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDR-P
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-P12-031

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Defense Threat Reduction Agency
8725 John J. Kingman Road STOP 6201
Fort Belvoir, VA 22060-6201

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
Published in Therapeutics, Pharmacology and Clinical Toxicology, Vol XVI, Number 4, 251-267, December 2012. This research was supported by the Defense Threat Reduction Agency – Joint Science and Technology Office, Medical S&T Division.

14. ABSTRACT
See reprint.

15. SUBJECT TERMS
Colinesterase reactivation, cyclosarin, methoxime, monoisonitrosoacetone, pralidoxime, sarin, VX

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Tsung-Ming Shih
UNLIMITED	UNLIMITED	UNLIMITED	UNLIMITED	17	**19b. TELEPHONE NUMBER** *(include area code)* 410-436-3414

Therapeutics, Pharmacology and Clinical Toxicology
Vol XVI, Number 4, December 2012
Pages: 251-267

ORIGINAL PAPERS

TERTIARY OXIMES ON BRAIN ACETYLCHOLINESTERASE AND CENTRAL EXCITATORY EFFECTS OF NERVE AGENTS

Shih Tsung-Ming*, Koenig A. Jeffrey, McDonough H. John

Pharmacology Branch, Research Division, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland 2100-5400, USA

Abstract. Organophosphorus nerve agents irreversibly inhibit the enzyme acetylcholinesterase (AChE), which leads to an excess of the cholinergic neurotransmitter acetylcholine in the synapses causing numerous toxic effects, including prolonged seizures and subsequent neuropathology. Current nerve agent therapies include pralidoxime (2-PAM) to reactivate inhibited AChE. The quaternary structure of this oxime does not allow it to cross the blood brain barrier (BBB) to reactivate brain AChE and to mitigate CNS toxicity. This study examined whether monoisonitrosoacetone (MINA) and N,N-diethyl-3-(2-(hydroxyimino)acetoxy) propan-1-aminium chloride (DHAP), two tertiary oximes that can penetrate the BBB, could prevent or reverse the central toxic effects of three nerve agents, sarin (GB), cyclosarin (GF), or VX, in guinea pigs. The first experiment tested whether MINA and DHAP could reactivate brain and peripheral tissue AChE inhibited by these nerve agents. Animals were challenged with a 1.0 x LD50 subcutaneous dose of a nerve agent and followed 15 min by one of 5 test doses of the oxime. Animals were euthanized 45 min after oxime treatment when blood and target tissues were collected. AChE activity was measured using the Ellman assay. MINA produced a dose-dependent AChE reactivation in both brain and peripheral tissues following all 3 nerve agents. DHAP reactivated GB-inhibited AChE in brain and peripheral tissues, but only GF-and VX-inhibited AChE in peripheral tissues. In a second experiment, the ability of MINA and DHAP to block or terminate nerve agent-induced electroencephalographic (EEG) seizure activity was evaluated. Animals instrumented to record brain EEG activity were challenged with a seizure-inducing dose (2.0 x LD50) of GB, GF, or VX, and oxime was administered one min after nerve agent exposure. MINA prevented or terminated seizures elicited by all three nerve agents. DHAP was effective against GB-induced seizures, but not against GF- or VX-induced seizures. Animals in which an oxime either prevented or terminated seizures almost invariably survived and lost significantly less body weight, when compared to those animals that experienced seizures that were not controlled by oxime treatment. In summary, the capacity of a tertiary oxime to reactivate nerve agent-inhibited AChE in the CNS was strongly associated with its ability to prevent or stop nerve agent-induced seizure activity. Therefore, reactivation of nerve agent-inhibited brain AChE using a CNS active oxime provides substantial therapeutic benefits and can reverse the neuro-excitatory sequelae of nerve agent intoxication.
Keywords: cholinesterase reactivation; cyclosarin; methoxime; monoisonitrosoacetone; pralidoxime; sarin; VX.

Abbreviations:
ACh, acetylcholine; **AChE**, acetylcholinesterase; **BBB**, blood brain barrier; **BCA**, bicinchoninic acid; **ChE**, cholinesterase; **CNS**, central nervous system; **DFP**, diisopropylfluorophosphates; **DHAP**, N,N-diethyl-3-(2-(hydroxyimino)acetoxy) propan-1-aminium chloride; **GB**, sarin; **GF**, cyclosarin; **LD50**, median lethal dose; **MINA**, monoisonitrosoacetone; **MMB-4**, methoxime; **OP**, organophosphorus compound; **2-PAM**, pralidoxime; **PB**, pyridostigmine bromide; **RBC**, red blood cell; **VX**, o-ethyl S-(2-(diisopropylamino)ethyl)methylphosphonothioate; WB, whole blood.

T.-M. Shih
U.S. Army Medical Research Institute of Chemical Defense
3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010-5400, USA
Tel.: +1 410 436 3414; fax: +1 410 436 2072
e-mail address: tsungming.a.shih@us.army.mil

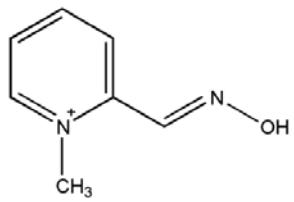
Introduction

Organophosphorus (OP) nerve agents, such as sarin (GB), cyclosarin (GF), and VX, are potent inhibitors of the cholinesterase (ChE) enzymes. Their toxic effects are due to hyperactivity of the cholinergic system as a result of ChE inhibition, especially acetylcholinesterase (AChE), and the subsequent increase in the level of the neurotransmitter acetylcholine (ACh) in the central nervous system (CNS) and periphery [1]. In the event of nerve agent poisoning, immediate medical therapy consists of an anticholinergic such as atropine sulfate to antagonize the effects of ACh at muscarinic receptors, an oxime such as pralidoxime (2-PAM), obidoxime (Toxogonin®), or HI-6 to reactivate any unaged, inhibited enzyme, and an anticonvulsant such as diazepam or midazolam to control motor convulsions and brain seizures [1-4]. The brain is a major target for the toxic effects of nerve agents. Inhibition of AChE in the brain results in prolonged seizures and neuropathology, and contributes to the incapacitating behavioral and lethal effects of these agents [5-7]. Reactivation of nerve agent-inhibited brain AChE may mitigate these CNS debilitating effects. The currently used oximes (i.e., 2-PAM, obidoxime, or HI-6) can reactivate ChEs in peripheral tissues but are quaternary nitrogen compounds with limited ability to cross the blood brain barrier (BBB). Their inability to enter the CNS and reactivate nerve agent-inhibited brain AChE is a major limitation of current oxime therapy. Thus, protecting and/or restoring AChE activity in the brain is an ultimate goal in total treatment of nerve agent intoxication.

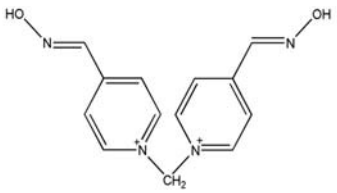
CNS-penetrating AChE reactivators for OP agent exposure have been investigated. Monoisonitrosoacetone (MINA) is a tertiary oxime that was investigated in the 1950s. It is highly lipid soluble, readily penetrates the BBB [8], and is capable of reactivating AChE within the CNS [8, 9]. Indeed, when used alone or in combination with atropine sulfate, MINA was shown to raise the LD_{50} doses of GB in several animal species [9-14]. Unfortunately, this oxime was not pursued further, because quaternary pyridinium oximes (e.g., 2-PAM) were reported to be more potent reactivators of AChE by several orders of magnitude in peripheral human erythrocytes [15]. We recently showed that MINA reactivated AChE in the brain, reduced toxic signs, improved survival, and prevented or terminated seizures following GB intoxication in guinea pigs [16-18]. The ability to reactivate AChE in the CNS would overcome the major limitation of the current oxime therapy for all OP compounds.

Studies by Benschop et al. [19] reported that the MINA analog N,N-diethyl-3-(2-(hydroxyimino) acetoxy) propan-1-aminium chloride (DHAP) demonstrated greater reactivating potency for GB-inhibited AChE *in vitro* than did MINA. Furthermore, they reported that DHAP exhibited the most pronounced CNS effectiveness among the aliphatic oximes (the class of oximes to which both DHAP and MINA belong) against OP compounds, diisopropyl fluorophosphate (DFP) and paraoxon, indicated by the hypothermia-reducing effect in rats. DHAP provided advantages over MINA due to its low toxicity, its relatively high reactivation efficiency of GB-inhibited AChE, and its PKa that is similar to that of 2-PAM [19].

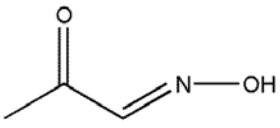
The present study was designed to evaluate and compare the efficacy of MINA and DHAP to reactive not only GB- but also GF- and VX-inhibited AChE in the brain and peripheral tissues and to prevent or block central excitatory consequences following GB, GF or, VX exposure in guinea pigs. Two quaternary oximes, 2-PAM and methoxime (MMB-4), were also included in this study for comparison (see Figure 1 for structures). 2-PAM



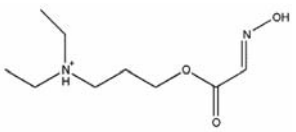
2-PAM



MMB-4



MINA



MMB-4

Figure 1. Chemical structures of oximes

is the standard oxime reactivator used in the U.S. for OP poisoning [3], whereas MMB-4, a broader spectrum *in vivo* reactivator, is a potential replacement for 2-PAM for therapy of OP nerve agent intoxication [20-22].

Materials and Methods

Subjects

Male Hartley guinea pigs (Crl:(HA) BR COBS) weighing 250-300 g were purchased from Charles River Labs (Kingston, NY). They were housed in individual cages in temperature (21 ± 2°C) and humidity (50 ± 10%) controlled quarters that were maintained on a 12-hour light – dark schedule (with lights on at 0600 h). Laboratory chow and filtered tap water were freely available whenever the animals were in home cages.

Materials

Saline (U.S.P.), Attane™ (Isoflurane, U.S.P.), and heparin sodium were purchased from Braun Medical, Inc. (Irvine, CA), Minrad, Inc. (Bethlehem, PA), and U.S.P., Inc. (Rockville, MD), respectively. PB (pyridostigmine bromide) was obtained from Hoffmann-La Roche Inc. (Nutley, NJ). Pralidoxime (2-PAM) was purchased from ScienceLab.com, Inc. (Houston, TX). MMB-4 dimethanesulfonate (methoxime; 1,1`-methylene-bis[4-(hydroxyimino) methyl] pyridinium dimethansulfonate) and DHAP (N,N-diethyl-3-(2-(hydroxyimino)acetoxy) propan-1-aminium chloride) were synthesized by the Southwest Research Institute (San Antonio, TX). MINA, acetylthiocholine iodide, atropine methyl nitrate (AMN), atropine sulfate, and AChE from electric eel were purchased from Sigma–Aldrich (St. Louis, MO). Bicinchoninic acid (BCA) Protein Assay Reagents A and B and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Pierce Biotechnology, Inc. (Rockford, IL). Sarin (GB; isopropylmethylphosphono fluoridate), cyclosarin (GF; cyclohexyl methylphosphonofluoridate), and VX (O-ethyl S-(2-(diisopropylamino)ethyl) methylphosphonothioate) were obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Nerve agents were diluted in saline prior to subcutaneous (s.c.) injection. Atropine and oxime compounds were prepared in saline individually for intramuscular (i.m.) injection. Injection volumes were 0.50 ml/kg for nerve agents and treatment drugs.

AChE reactivation experiment

For the purpose of comparing the AChE reactivation capability among oximes, the equivalent of 1.0 times the median lethal dose (LD_{50}) of each nerve agent was used. The duration of the interaction between oxime and the agent-inhibited enzyme was set at 45 min. The dose of 2-PAM (25 mg/kg, or 145 μmol/kg) used was equivalent to three autoinjector doses (as in Mark I Nerve Agent Antidote Kit) for a 70 to 75 kg person. The dose of MMB-4 was 26 mg/kg (58.0 μmol/kg, i.m.), which was equivalent to the maximum three autoinjector doses to be given to a 70 to 75 kg person (based on HI-6 Dichloride; [23]). Tertiary oximes were evaluated in a dose-dependent manner, based on their reported toxic doses and solubility in water [19]. In general, the 5 doses within each oxime were separated by a constant logarithmic interval.

General procedures

One to 3 days prior to the experiment, control blood samples (~0.25 ml) were drawn using the toenail clip method [24] and collected into a 1.0-ml microfuge tube containing 50 μl of heparin sodium (15 U/ml) to determine baseline AChE activity in whole blood (WB) and red blood cells (RBC). On the day of the study, guinea pigs were pretreated with atropine methyl nitrate (AMN; 1.0 mg/kg, i.m.) 15 min prior to nerve agent exposure to minimize peripheral toxic effects. AMN is a peripherally acting muscarinic receptor blocker that does not affect AChE activity. Animals were injected s.c. with either saline (0.5 ml/kg) or a 1.0 x LD_{50} dose of GB (42.0 μg/kg), GF (57.0 μg/kg), or VX (8.0 μg/kg). Fifteen min after nerve agent injection, when the inhibition of AChE activity by these nerve agents reached maximum [25], saline (0.5 ml/kg), MINA (12.6, 22.1, 35.0, 55.5, 87.9, or 139.3 mg/kg), DHAP (35.5, 50.0, 70.8, 100.0, or 141.3 mg/kg), 2-PAM (25.0 mg/kg), or MMB-4 (26.0 mg/kg) was given i.m. Control animals received s.c. saline (no nerve agent) and i.m. saline (no oxime).

The severity of toxic signs of each animal was scored at 13 min after nerve agent (just before oxime treatment) and again at 58 min after nerve agent injection (about 43 min after oxime treatment and prior to sample collection). Sixty min after s.c. saline or nerve agent administration, the animals were deeply anesthetized with isoflurane and euthanized by decapitation. Blood (~0.25 ml) was collected into a 1.0 ml microfuge tube as described above to determine AChE activity in WB and RBC. Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord and striatum) and peripheral tissue (diaphragm, heart, and skeletal muscle) were dissected and processed for AChE activity on a separate day. For the WB samples, 20 μl of blood was diluted 1:25 (v:v) in 1% Triton X-100 solution. For the RBC samples, the original blood sample was centrifuged for 5 min at 14,000 rpm, and 10 μl of the packed RBC was then diluted 1:50 in 1% Triton X-100 solution. Brain

Table I.*(continue on the next page)* lithium values obtained from control group

samples were diluted 1:20 (w:v), while peripheral samples were diluted 1:5, in 1% Triton X-100 solution and then homogenized. The homogenates were then centrifuged (31,000 x g at 4°C; 20 min for brain and 30 min for peripheral tissues), and the supernatant was decanted and stored at -80°C until AChE activity and protein concentration analyses were performed.

Toxic signs test

At 13 and 58 min after GB, GF, or VX injection, guinea pigs were observed for signs of cholinergic toxicity [16]. This toxic sign score system used for guinea pigs was modified from that for rats reported by Shih and Romano [26]. They were scored first for absence (0) or presence (1) of each of the following signs: salivation, lacrimation, and nystagmus. Then, general motor signs were assigned a 0-3 score: normal = 0, fasciculation = 1, tremor = 2, or convulsion = 3. Next, the guinea pig was allowed to walk on the bench top and general state was assigned a 0-3 score: normal = 0, mild uncoordination = 1, impaired movement/with righting reflex = 2, or prostration/no righting reflex = 3. A cumulative score was then calculated by tabulating the salivation, lacrimation, nystagmus, general motor and general state score for each subject. The maximal attainable score was 9. A cumulative score was categorized as mild (scores = 1-3), moderate (scores = 4-6), or severe intoxication (scores = 7-9).

AChE activity assay

AChE activity in the blood, brain, and peripheral tissues was measured with a modified colorimetric Ellman assay [27] according to methods described by Shih et al. [22, 25]. For brain and peripheral tissues, protein concentration was determined using the BCA protein assay. AChE activity in these tissues was expressed as μmol of substrate hydrolyzed/g protein/min. For blood, AChE activity was expressed as μmol of substrate hydrolyzed/ml/min. The AChE activity was then expressed as percent of the saline/saline control group for brain and peripheral tissue samples and percent of the individual baseline AChE activity for the blood obtained prior to the nerve agent experiment.

Anticonvulsant experiment

All animals, while under isoflurane anesthesia, were prepared for recording of electroencephalogram (EEG) approximately one week before experimentation by implanting cortical stainless steel screw electrodes using previously described procedures [6, 28]. On the day of the experiment, guinea pigs were placed in individual recording chambers and continuously monitored for EEG activity. EEG recordings were collected using amplifiers and software supplied by Neurodata, Inc. (Pasadena, CA; low frequency filter = 0.3 Hz; high frequency filter = 40 Hz; sampling rate = 128 Hz). After a 30 min baseline EEG recording, animals received pyridostigmine bromide (PB, 0.026 mg/kg, i.m.) 30 min before challenged s.c. with 2 x LD_{50} of GB (84 μg/kg), GF (114 μg/kg), or VX (16 μg/kg). One min after nerve agent challenge, the animal was treated i.m. with atropine sulfate (0.5 mg/kg) and a dose of MINA, DHAP, 2-PAM or MMB-4. The doses of MINA included: 18.0, 32.0, 42.2, or 56.0 mg/kg for GB exposure, 56.0, 75.0, 100.0, or 133.0 mg/kg for GF exposure, and 18.0, 32.0, 42.2, 56.0, 100.0, or 133.0 mg/kg for VX exposure. The doses of DHAP given were 32.0, 56.0, 75.0, 100.0, 133.0, or 180.0 mg/kg for GB exposure, 133.0 or 180.0 mg/kg for GF exposure, and 100.0, 133.0, or 180.0 mg/kg for VX exposure. Initially, the quaternary oxime 2-PAM (25 mg/kg) was included for comparison against all 3 nerve agents. MMB-4 (26 mg/kg) was used in the comparison study. However, when MMB-4 showed no anticonvulsant effectiveness against GB, the studies with GF and VX were discontinued. Animals were observed continuously for the first hour following nerve agent exposure and periodically thereafter for at least 4 hr. EEG was recorded continuously throughout this time and again for another 30 min at 24 hr after exposure. Seizure onset was operationally defined as the appearance of ≥ 10 sec of rhythmic high amplitude spikes or sharp wave activity in the EEG tracing. Each animal was rated as having the seizure terminated (OFF) or not terminated (NOT OFF) based on the overall appearance of the EEG record at the end of the experimental day and during the 30 min observation at 24 hr. An animal was rated as OFF only if the seizure was terminated and the EEG remained normal at all subsequent observation times. Mortality was recorded 24 hr after nerve agent exposure. Animals that survived 24 hr were euthanized, and their brains were processed for histopathological evaluation.

Data analysis

For the AChE reactivation experiment.

Statistical analysis of toxic sign scores was performed using a Kruskal-Wallis test to compare across treatments. A Dunn's test was then performed as a post-test for multiple comparisons. Differences in the incidence of toxic signs between treatment groups were evaluated using Fisher's Exact Test. A Mann-Whitney test was performed to compare toxic sign scores between the 13 min and 58 min scoring times.

For AChE activity, statistical analysis was performed using a one-way analysis of variance

(ANOVA) to compare across treatment groups for each nerve agent. A post-hoc Tukey test was used for multiple comparisons. Statistical significance is defined as $p \leq 0.05$.

To avoid presenting the AChE dataset for 2-PAM and MINA in table forms, since these data have been published elsewhere [29], and to compare the AChE reactivation data among the 4 oximes, we analyzed these data further by converting them to percent of control activity and then calculating the actual percent of AChE recovery (via reactivation) in oxime-treated groups from that of nerve agent control group. The significantly increased AChE activity (via reactivation) in oxime-treated groups was calculated and expressed as the percent recovery of the saline/saline-treated control baseline activity above that of the remaining AChE activity in the nerve agent-inhibited group (i.e., % AChE recovery = the % of control AChE activity in nerve agent-exposed and oxime-treated group minus the % of control AChE activity in nerve agent-exposed and saline-treated group), as shown in Figure 2.

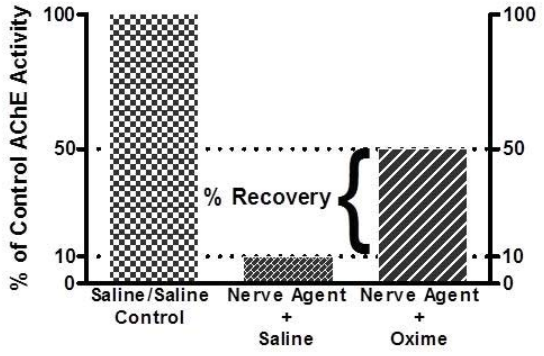


Figure 2. Calculation and expression of % AChE recovery produced by oxime

For the anticonvulsant experiment.

Dose-effect curves and the median effective dose (ED_{50}) for anticonvulsant activity of each oxime were determined by probit analysis [30] using two to six doses with two to seven animals per group. A probit regression analysis (SPSS for Windows, Version 17.0, Chicago, IL) was used to estimate the ED_{50} values along with the 95% confidence intervals for each oxime treatment and nerve agent combination. Seizure onset time, time to seizure termination, and body weight loss between treatment groups were compared using a one-way ANOVA with a post-hoc Tukey test. Statistical significance for all tests was defined as $p \leq 0.05$.

Results

AChE reactivation experiment

Signs of toxicity and lethality.

Under the conditions of this study, guinea pigs exposed to a 1.0 x LD_{50} of GB, GF, or VX and not receiving oxime therapy exhibited typical signs of nerve agent intoxication, such as salivation, rhinorrhea, tremor, muscle fasciculations, and convulsions. These animals showed a high incidence of cholinergic toxic signs: GB, 88% (15 of 17 animals); GF, 100% (7 of 7 animals); and VX, 78% (7 of 9 animals). The average toxic sign scores for GB- and VX-exposed saline-treated animals significantly increased from the 13 min (2.40 ± 0.6 [N=10] and 0.33 ± 0.24 [N=9], respectively) to the 58 min (5.00 ± 0.59 [N=13] and 3.44 ± 0.75 [N=9], respectively) scoring time, indicating moderate intoxication. Toxic sign scores for GF-exposed saline-treated animals also rated in the moderate range, but did not significantly increase from the 13 min (4.60 ± 0.48 [N=10]) to the 58 min (5.50 ± 0.22 [N=10]) scoring time. Thus, the development and progression of toxic signs were fastest for GF, followed by GB, and slowest for VX. No animal treated with 2-PAM, MMB-4, MINA, or DHAP died within 60 min of GB, GF or VX exposure under the conditions of this study.

Animals treated with 2-PAM (25 mg/kg) and MMB-4 (26 mg/kg) showed 100% incidence of toxic signs following GB (8/8 and 7/7, respectively) and GF (8/8 and 8/8, respectively) exposure, while only 38% (3/8) and 63% (5/8), respectively, displayed toxic signs following VX exposure. When comparing the average toxic sign scores at 58 min, 2-PAM or MMB-4 treatment did not mitigate the severity of toxic signs following GB, GF, or VX exposure, with one exception. The average toxic sign scores at 58 min for VX-exposed 2-PAM-treated animals were significantly reduced from 3.44 ± 0.75 (N=9) in saline-treated animals to 0.75 ± 0.37 (N=8), reducing the severity of intoxication from moderate to mild.

The number of GB-exposed animals treated with MINA showing signs of nerve agent intoxication (48%, 19 of 40 animals) were significantly smaller than that of the saline-treated controls (88%). A reduction in the incidence of toxic signs following VX exposure in MINA-treated animals (47%, 26/55) was also observed, but was not found to be significant in comparison with saline-treated controls (78%, 7/9). The incidence (95%, 38/40) of toxic signs in animals treated with MINA following a 1.0 x LD_{50} of GF was similar to that of the saline-treated controls (100%). Additionally, animals treated with MINA at doses of 35.0 mg/kg and above displayed significantly lower toxic sign scores (from 0.38 ± 0.26 [N=8] to 1.00 ± 0.73 [N=8])

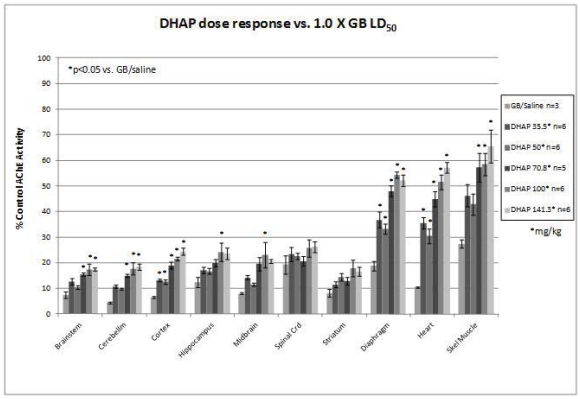
following GB exposure than did the saline-treated controls (5.00±0.59 [N=13]). Animals treated with MINA at doses of 55.5 mg/kg and above displayed significantly lower toxic sign scores (from 2.00±0.42[N=8] to 3.14±0.51 [N=7]) following GF exposure than did saline-treated controls (5.50±0.22 [N=10]). In the case of VX exposure, as the doses of MINA were increased from 22.1 mg/kg and above, the toxic scores (ranging from 0.73±0.33 [N=11] to 0.82±0.44 [N=11]) were significantly reduced from saline-treated controls (3.44+0.75 [N=9]). In some of the guinea pigs toxic signs were observed with MINA alone at the high dose (139.34 mg/kg).

The incidences of cholinergic toxic signs in animals treated with DHAP following GB, GF, and VX exposure were 70% (21/30), 100% (30/30), and 83% (25/30), respectively. Following GB exposure and DHAP treatment, there was a trend of reduced toxic signs scores as the doses were increased from 70.8 mg/kg (scores ranging from 2.00±0.26 [N=6] to 1.17±0.60 [N=6]) when compared with the scores for saline-treated controls (3.44±0.75 [N=9]). However, these reductions in toxic scores were not statistically significant. No other reductions of toxic sign scores were observed at any doses of DHAP following GF or VX exposure.

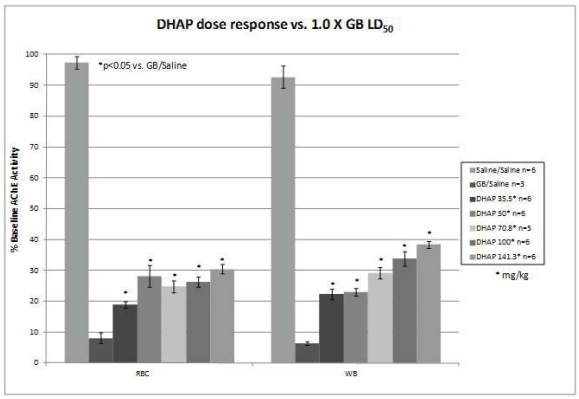
Reactivation of AChE activity.

MINA was capable of reactivating AChE activity inhibited by all three nerve agents in a dose dependent manner as described elsewhere [29]. Following GB exposure, doses of MINA at 55.5 mg/kg and above were able to significantly reactivate inhibited brain AChE. As the dose of MINA increased the AChE activity was reactivated in more brain regions. At the highest dose of 139.3 mg/kg, all seven brain regions displayed significant AChE reactivation. Very similar results were obtained following GF and VX exposures. The relative brain AChE reactivating potency of MINA against these nerve agents was GB > GF > VX. MINA reactivated AChE inhibited by GB and GF in at least two peripheral tissues when the dose reached 55.5 mg/kg and above. MINA reactivated VX-inhibited AChE in one peripheral tissue at 55.5 mg/kg and in all three tissues at 139.3 mg/kg. In the blood components (RBC and WB) MINA reactivated GB-inhibited AChE in the blood at doses 55.5 mg/kg and above, while only reactivating VX-inhibited AChE in the blood at 139.3 mg/kg. At 35.0 mg/kg and above, MINA reactivated GF-inhibited AChE only in the WB. The capability of MINA to reactivate AChE activity in peripheral tissues and blood inhibited by these three nerve agents was also in the order of GB > GF > VX.

On the other hand, DHAP only reactivated AChE activity inhibited by GB in several brain regions (Figure 3). DHAP reactivated GB-inhibited AChE in the cortex at low doses (35.5 and 50.0 mg/kg), added in the brainstem and cerebellum at 70.8 mg/kg, and then included hippocampus and midbrain at 100 mg/kg. No enzyme reactivation was observed in the striatum and spinal cord regions (Figure 3A). DHAP was very effective in reactivating AChE in the peripheral tissues and blood at all doses following exposure to GB (Figure 3B). Following exposure to GF and VX, DHAP was not able to reactivate AChE in any brain regions, but showed reactivation only in skeletal muscle, RBC or WB at doses 70.8 mg/kg or higher (data not shown).



A



B

Figure 3. Dose-response effects of DHAP on GB-inhibited AChE activity in brain regions, peripheral tissues, and blood

We evaluated these AChE reactivation data sets further by two different ways after subjecting them initially to standard statistical procedure as described earlier to identify significant treatment groups.

Evaluation by the percentage of significant recovery of AChE activity.

We evaluated the percent of recovery of AChE activity by MMB-4 and 2-PAM at a single dose and

A. Following sarin exposure

Sarin (GB)				
Oxime	Dose Range (mg/kg)	Significant AChE % Recovery		
		Blood	Peripheral Tissues	Brain Regions
MMB-4	26.0	64.0 - 69.2	40.8 - 51.7	--
2-PAM	25.0	53.1 - 58.3	29.4 - 50.0	--
MINA	22.1 - 139.3	11.1 - 34.4	10.0 - 24.1	14.5 - 31.2
DHAP	35.5 - 141.3	10.9 - 32.0	14.6 - 46.8	6.1 - 17.9

B. Following cyclosarin exposure

Cyclosarin (GF)				
Oxime	Dose Range (mg/kg)	Significant AChE % Recovery		
		Blood	Peripheral Tissues	Brain Regions
MMB-4	26.0	42.4 - 47.6	15.2 - 30.6	19.8[c]
2-PAM	25.0	11.9[a]	--	--
MINA	22.1 - 139.3	13.8 - 37.3	11.7 - 34.7	8.1 - 16.7
DHAP	35.5 - 141.3	3.8 - 8.2	9.0 - 12.0	--

C. Following VX exposure

VX				
Oxime	Dose Range (mg/kg)	Significant AChE % Recovery		
		Blood	Peripheral Tissues	Brain Regions
MMB-4	26.0	58.4 - 67.6	13.2 - 34.6	--
2-PAM	25.0	30.0 - 37.9	17.8 - 22.3	--
MINA	22.1 - 139.3	15.0 - 33.9	8.1 - 21.8	10.4 - 23.2
DHAP	35.5 - 141.3	8.4 - 10.9	15.4[b]	--

Table I. Percentage of control AChE activity recovery in blood, peripheral tissue, and brain regions by oxime treatments at various doses following exposure to sarin, cyclosarin, and VX[1]

[1] Guinea pigs were pretreated with atropine methyl nitrate (2.0 mg/kg, i.m.) 15 min prior to challenge with a subcutaneous dose (1.0 x LD50) of a nerve agent (GB, GF, or VX), followed 15 min later with an intramuscular dose of the oxime. Tissues were collected for AChE determination at 60 min after nerve agent challenge.

Data in each cell show the statistically significant ranges of % of AChE recovery in three tissue components (blood, peripheral tissues, and brain regions) following oxime treatment at various doses from low to high doses listed.

[a,b,c,d,e] indicate significant AChE reactivation in a single tissue: [a]WB; [b]skeletal muscle; and [c]spinal cord.

-- (double dash line) indicated there was no significant AChE reactivation in the tissue component by oxime treatment.

by MINA and DHAP at various doses following exposure to GB, GF, and VX in blood, peripheral tissues, and brain regions. Tables IA, IB, and IC summarize this evaluation following exposure to GB, GF, and VX, respectively.

Following GB exposure (Table IA), the significant percentage of AChE recovery in the blood and peripheral tissues for MMB-4 treatment was 64.0 – 69.2 % and 40.8 – 51.7 %, respectively, and for 2-PAM treatment was 53.1 – 58.3 % and 29.4 – 50.0 %, respectively. These 2 quaternary oximes are highly effective in reactivating peripheral AChE. No AChE reactivation in the CNS, however, was observed for these two oximes. In the blood, the maximum percentage of AChE recovery for MINA and DHAP was similar but was only one-half that of 2-PAM or MMB-4. In the peripheral tissues, the maximum percentage of AChE recovery for DHAP was comparable with that of 2-PAM or MMB-4, while the AChE recovery for MINA was only one-half that of the other three oximes tested. In the CNS both MINA and DHAP showed AChE reactivation. However, the percentage of AChE recovery for MINA was two-fold higher than that for DHAP. When comparing the reactivating potencies between peripheral and CNS compartments, DHAP displayed higher reactivating potency in the periphery than in the CNS, while MINA showed comparable potency between these two tissue compartments.

Following GF exposure (Table IB), MMB-4 was able to reactivate AChE in the blood, peripheral tissues, and the spinal cord with 42.4 – 47.6 %, 15.2 – 30.6 %, and 19.8 % recovery of AChE activity, respectively. However, 2-PAM showed significant AChE reactivation only in the WB with an 11.9% recovery of enzyme activity. In the blood and peripheral tissues, MINA showed similar percentages of AChE recovery to those of MMB-4, while DHAP produced only 8.2 to12.0 % reactivation of AChE activity. Although both MINA and DHAP were capable of reactivating AChE in the blood and peripheral tissues, only MINA reactivated AChE in the brain tissues with a maximum of 16.7% recovery.

A. Quaternary oxime treatment*

	2-PAM (25.0)	MMB-4 (26.0)
(a) Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord, striatum)		
GB	-	-
GF	-	-
VX	-	-
(b) Peripheral tissues (diaphragm, heart, skeletal muscle)		
GB	+++	+++
GF	-	+++
VX	++	+++
(c) Blood (red blood cells and whole blood)		
GB	++	++
GF	+	++
VX	++	++

B. MINA treatment*

	MINA (22.1)	MINA (35.0)	MINA (55.5)	MINA (87.9)	MINA(139.3)
(a) Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord, striatum)					
GB	-	+	+++++	++++++	+++++++
GF	-	-	++	++	++++++
VX	-	-	+	+	+++++
(b) Peripheral tissues (diaphragm, heart, skeletal muscle)					
GB	-	-	++	++	+++
GF	-	+	++	++	++
VX	-	-	+	+	+++
(c) Blood (red blood cells and whole blood)					
GB	-	-	+	++	++
GF	-	+	+	+	+
VX	-	-	-	-	++

C. DHAP treatment*

	DHAP (35.5)	DHAP (50.0)	DHAP (70.8)	DHAP (100.0)	DHAP (141.3)
(a) Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord, striatum)					
GB	+	+	+++	+++++	+++
GF	-	-	-	-	-
VX	-	-	-	-	-
(b) Peripheral tissues (diaphragm, heart, skeletal muscle)					
GB	++	++	+++	+++	+++
GF	-	-	-	+	+
VX	-	-	+	-	-
(c) Blood (red blood cells and whole blood)					
GB	++	++	++	++	++
GF	-	-	+	+	+
VX	-	-	-	++	+

Table II. Number of tissues in brain, periphery and blood showing significantly increased AChE activity after oxime treatment at various doses following exposure to saline (GB), cyclosarin (GF), and VX[1,2]

* oxime dose in mg/kg, i.m.

[1] Guinea pigs were pretreated with methylatropine nitrate (2.0 mg/kg, i.m.) 15 min prior to challenge with a subcutaneous dose (1.0 x LD50) of a nerve agent (GB, GF, or VX), followed 15 min later with an intramuscular dose of the oxime. Tissues were collected for AChE determination at 60 min after nerve agent challenge.

[2] The significant AChE reactivation data were divided into three tissue compartments: the brain regions, the peripheral tissues, and the blood components. A "+" sign was assigned for each of the brain regions, peripheral tissues, or blood components when an oxime treatment significantly reactivated nerve agent-inhibited AChE. For example, if an oxime treatment significantly reactivated AChE in all seven brain regions its cell received the maximum of seven "+" signs; in all three peripheral tissues its cell received the maximum of three "+" signs; or in the two components of blood its cell received two "+" signs. When an oxime treatment did not significantly reactivate a nerve agent-inhibited AChE in any tissue, its cell was assigned a "-" sign.

Following VX exposure (Table IC), the significant percentage of AChE recovery in the blood and peripheral tissue for MMB-4 treatment was 58.4 – 67.6 % and 13.2 – 34.6 %, respectively, and for

2-PAM treatment, 30.0 – 37.9 % and 17.8 – 22.3 %, respectively. Both quaternary oximes are highly effective in reactivating peripheral AChE. No AChE reactivation in the CNS, however, was observed for these 2 oximes. MINA was able to reactivate AChE to the same degree as did 2-PAM in the blood and peripheral tissues, while DHAP reactivated AChE only in the blood and skeletal muscle with much less effectiveness. Additionally, MINA was able to reactivate VX-inhibited AChE in the CNS to the same degree as in the blood and peripheral tissues.

Evaluation by the number of tissues showing significant recovery of AChE activity.

We also evaluated the AChE reactivation data for the numbers of tissues showing significant recovery of AChE activity by dividing them into three tissue compartments: the brain regions, the peripheral tissues, and the blood components. A "+" sign was assigned for each of the seven brain regions, three peripheral tissues, or two blood components when an oxime treatment significantly reactivated nerve agent-inhibited AChE. Table II summarizes the statistically significant increases in AChE activity resulting from 2-PAM and MMB-4 (2A), MINA (2B), and DHAP (2C) treatment following exposure to GB, GF, and VX in terms of the number of tissues in the brain regions, the peripheral tissues, and the blood components. Both 2-PAM and MMB-4 showed no AChE reactivation in any brain region (Table IIA). Although 2-PAM was capable of reactivating AChE in GB- and VX-inhibited enzymes in the peripheral tissues and blood, it was ineffective in GF-inhibited AChE in these samples with the exception of the WB. In contrast, MMB-4 was capable of reactivating AChE inhibited by GF in all blood and peripheral tissues regardless of nerve agent.

It is striking to notice that MINA was capable of reactivating AChE inhibited by all three nerve agents in at least 5 brain regions (Table IIB). Following GB exposure, doses of MINA at 55.5 mg/kg and above were able to significantly reactivate inhibited brain AChE. As the dose of MINA increased, the reactivation of AChE increased in additional brain regions. At the highest dose of 139.3 mg/kg, all seven brain regions displayed significant AChE reactivation. MINA also began to show its ability to reactivate GF-inhibited AChE when the dose reached 55.5 mg/kg and above, and at 139.3 mg/kg, all brain regions with the exception of the striatum, showed significant AChE reactivation. In the case of VX exposure, MINA at doses of 55.5 mg/kg significantly reactivated AChE only in the hippocampus. When the dose reached 139.3 mg/kg, a total of five brain regions (brainstem, cerebellum, cortex, hippocampus, and midbrain) displayed significant AChE reactivation. The relative brain AChE reactivating potency of MINA against these nerve agents was GB > GF = VX. In the peripheral tissues MINA reactivated AChE inhibited by GB and VX in all three tissues at 139.3 mg/kg, and GF-inhibited AChE in 2 tissues (diaphragm and heart). In the blood components, MINA reactivated GB- and VX-inhibited AChE in both components at 139.3 mg/kg, while at 35.0 mg/kg and above, MINA reactivated GF-inhibited AChE only in the WB. The capability of MINA to reactivate AChE activity in peripheral tissues and blood inhibited by these three nerve agents was in the order of GB > VX = GF.

DHAP (Table IIC), at 100 mg/kg, reactivated GB-inhibited AChE in 5 brain regions, with the exception of the striatum and spinal cord. It was also very effective in reactivating AChE in the peripheral tissues and blood at all doses following exposure to GB. Following exposure to GF, DHAP was not able to reactivate AChE in any brain regions, and showed reactivation only in the skeletal muscle and WB at doses 100.0 mg/kg or higher. It was, however, effective in reactivating AChE inhibited by VX in both blood components but not effective in reactivating AChE in the peripheral or CNS tissues. Therefore, DHAP was predominately effective only in reactivation of GB-inhibited AChE.

Anticonvulsant experiment

Seizure occurrence and survival at 24 hr.

The incidences of EEG seizure occurrence and 24 hr lethality following exposure to 2.0 x LD_{50} nerve agents and treatments with quaternary and tertiary oximes are shown in Table III. All guinea pigs (100%) exposed to 2.0 x LD_{50} GB and treated with atropine sulfate plus 2-PAM (Table IIIA) or MMB-4 (Table IIIB) developed continuous seizure activity (*status epilepticus*), and only 60% and 20% of animals, respectively, survived 24 hr. When treated with MINA at doses of 42 and 56 mg/kg resulted in 67 and 100% of animals, respectively, never exhibiting EEG seizure activity. At 24 hr, 17, 50, 100, and 100% of these animals survived with MINA doses of 18, 32, or 42 and 56 mg/kg, respectively (Table IIIB). With DHAP treatment at doses of 32, 56, 75, and 100, 133, or 180 mg/kg, all animals exhibited EEG seizure activity, and 50, 67, and 100% of these animals, respectively, survived 24 hr (Table IIIC).

All animals (100%) exposed to 2.0 x LD_{50} GF and treated with atropine sulfate plus 2-PAM developed *status epilepticus*, and none of these animals survived 24 hr (Table IIIA). With MINA treatment at doses of 56, 75, 100, and 133 mg/kg, 0, 25, 33, and 40% of animals, respectively, never exhibited

A. 2-PAM and MMB-4 treatment

	GB			GF			VX		
Oxime Dose (mg/kg)	Seizure Occurrence	Anticonvulsant Response	24 hr Lethality	Seizure Occurrence	Anticonvulsant Response	24 hr Lethality	Seizure Occurrence	Anticonvulsant Response	24 hr Lethality
2-PAM (25)	10/10	0/10	4/10	10/10	0/10	10/10	8/8	0/8	8/8
MMB-4 (26)	5/5	0/5	4/5	--	--	--	--	--	--

B. MINA treatment

	GB			GF			VX		
Oxime Dose (mg/kg)	Seizure Occurrence	Anticonvulsant Response	24 hr Lethality	Seizure Occurrence	Anticonvulsant Response	24 hr Lethality	Seizure Occurrence	Anticonvulsant Response	24 hr Lethality
18.0	6/6	0/6	5/6	--	--	--	2/2	0/2	1/2
32.0	6/6	2/6	3/6	--	--	--	2/2	0/2	2/2
42.2	2/6	5/6	0/6	--	--	--	5/5	0/5	5/5
56.0	0/6	6/6	0/6	4/4	0/4	2/4	4/5	2/5	4/5
75.0	--	--	--	3/4	2/4	2/4	--	--	--
100.0	--	--	--	4/6	4/6	1/6	3/6	4/6	2/6
133.0	--	--	--	3/5	5/5	0/5	5/7	6/7	1/7

C. DHAP treatment

	GB			GF			VX		
Oxime Dose (mg/kg)	Seizure Occurrence	Anticonvulsant Response	24 hr Lethality	Seizure Occurrence	Anticonvulsant Response	24 hr Lethality	Seizure Occurrence	Anticonvulsant Response	24 hr Lethality
32.0	2/2	0/2	1/2	--	--	--	--	--	--
56.0	4/4	0/4	2/4	--	--	--	--	--	--
75.0	4/4	0/4	2/4	--	--	--	--	--	--
100.0	6/6	1/6	3/6	--	--	--	2/2	0/2	2/2
133.0	6/6	1/6	2/6	4/4	0/4	4/4	6/6	1/6	6/6
180.0	5/5	4/5	0/5	3/3	0/3	3/3	4/6	4/6	2/6

Table III. Seizure occurrence, anticonvulsant response and 24 hr lethality following nerve agent exposure and oxime treatment[1]

[1] Guinea pigs received pyridostigmine bromide (0.026 mg/kg, i.m.) 30 min before receiving nerve agents (2.0 x LD50), followed 1 min later by atropine sulfate and oxime treatment.
-- Not tested

		Seizure	Deaths	Non-seizure*	Deaths
GB	2-PAM	10	4	0	--
	MMB-4	5	4	0	--
	MINA	11	7	13	1
	DHAP	21	10	6	0
	Sub-total	47	25 (53%)	19	1 (5%)
GF	2-PAM	10	10	0	--
	MINA	8	5	11	0
	DHAP	7	7	0	--
	Sub-total	25	22 (88%)	11	0 (0%)
VX	2-PAM	8	8	0	--
	MINA	15	13	12	2
	DHAP	9	9	5	1
	Sub-total	32	30 (94%)	17	3 (18%)
Total		104	77 (74%)	47	4 (9%)

Table IV. Number of animals per seizure status and the number of deaths in each group

* seizure either prevented or terminated.
-- (double dash line) indicated there was no death to be recorded.

EEG seizure activity, and 50, 50, 83, and 100% of these animals, respectively, survived 24 hr (Table IIIB). When treated with DHAP at doses of 133 and 180 mg/kg, all (100%) animals exhibited EEG seizure activity, and none of these animals survived 24 hr (Table IIIC).

All animals (100%) exposed to 2.0 x LD_{50} VX and treated atropine sulfate plus 2-PAM developed *status epilepticus*, and none of these animals survived 24 hr (Table IIIA). When treated with MINA at doses of 56, 100, and 133 mg/kg reduced occurrence of EEG seizure activity (20, 50, and 29% of animals, respectively) and increased survival (10, 67, and 86% of these animals, respectively) at 24 hr (Table IIIB). Only the highest dose of DHAP (180 mg/kg) reduced the occurrence of EEG seizure activity (67%) and increased survival (33%) at 24 hr (Table IIIC).

Relationship between seizure and lethality.

The relationship between seizure activity and lethality following nerve agent exposure was evaluated from the results described above and summarized in Table IV. There was a strong relationship between the control of seizure activity and protection against nerve agent-induced lethality. When animals were exposed to 2.0 x LD_{50} GB, GF, and VX, 53%, 88%, and 94%, respectively, of the animals died, if the oxime treatments failed to prevent or stop seizures. However, if the seizures were prevented or stopped by oxime treatments, only 5%, 0%, and 18%, respectively, of the animals died. Overall, only 9% (4/47) of the animals that had their seizures prevented or terminated by the oxime treatment died within 24 hr, while 74% (77/104) of the animals died within 24 hr when oxime treatment failed to prevent or stop seizures. This difference was highly significant (χ^2 = 95.8, df=1, p<0.001) and was consistent across either the individual nerve agents or the individual oxime treatments.

Seizure onset.

Following 2.0 x LD_{50} GB exposure the seizure onset times for 2-PAM- and MMB-4-treated animals were 7.1±0.4 (N=10) and 6.0±0.4 (N=5) min, respectively (Table V). In those animals that displayed EEG seizure activity, the average seizure onset times for MINA- and DHAP-treated groups after GB were 7.0±0.7 (N=14) and 6.0±0.5 (N=27) min, respectively (Table V). There was no significant difference in time to seizure onset among these four oxime treatment groups.

Following 2.0 x LD_{50} GF exposure the seizure onset time for 2-PAM-treated animals was 5.8±0.5 (N=10) min (Table V). In those animals that displayed EEG seizure activity, the average seizure onset times for MINA- and DHAP-treated groups after GF were 8.1±2.3 (N=14) and 7.7±1.5 (N=7) min, respectively (Table V). There was no significant difference in time to seizure onset among these oxime treatment groups for either GB or GF exposure.

Following 2.0 x LD_{50} VX exposure the seizure onset time for 2-PAM-treated animals was 15.3±1.0 (N=8) min (Table V). In those animals that displayed EEG seizure activity, the average seizure onset times for MINA- and DHAP-treated groups after VX were 30.7±2.6 (N=21) and 20.4±1.4 (N=12) min, respectively (Table V). Time to seizure onset for MINA treatment group was significantly different from both 2-PAM and DHAP treatment groups (p<0.05).

	GB				GF			VX		
Oxime Dose (mg/kg)	MINA	DHAP	2-PAM	MMB-4	MINA	DHAP	2-PAM	MINA	DHAP	2-PAM
18.0	6.2 ± 0.6 (6)	--	--	--	--	--	--	21.0 ± 3.8 (2)	--	--
25.0	--	--	7.1 ± 0.4 (10)	--	--	--	5.8 ± 0.5 (10)	--	--	15.3 ± 1.0 (8)
26.0	--	--	--	6.0 ± 0.4 (5)	--	--	--	--	--	--
32.0	7.7 ± 1.5 (6)	5.2 ± 1.0 (2)	--	--	--	--	--	29.6 ± 10.3 (2)	--	--
42.2	7.0 ± 1.4 (2)	--	--	--	--	--	--	26.9 ± 1.4 (5)	--	--
56.0	--	6.7 ± 2.5 (4)	--	--	4.9 ± 0.8 (4)	--	--	24.0 ± 3.3 (4)	--	--
75.0	--	5.5 ± 0.5 (4)	--	--	6.9 ± 0.5 (3)	--	--	--	--	--
100.0	--	7.3 ± 1.4 (6)	--	--	5.7 ± 1.2 (4)	--	--	36.9 ± 9.0 (3)	18.3 ± 0.3 (2)	--
133.0	--	5.2 ± 0.3 (6)	--	--	16.8 ± 10.2 (3)	5.7 ± 1.3 (4)	--	40.5 ± 6.5 (5)	18.8 ± 1.5 (6)	--
180.0	--	5.4 ± 0.4 (5)	--	--	--	10.3 ± 2.7 (3)	--	--	23.9 ± 3.0 (4)	--
Mean	7.0 ± 0.7 (14)	6.0 ± 0.5 (27)	7.1 ± 0.4 (10)	6.0 ± 0.4 (5)	8.1 ± 2.3 (14)	7.7 ± 1.5 (7)	5.8 ± 0.5 (10)	30.7 ± 2.6 (21)*[a]	20.4 ± 1.4 (12)*	15.3 ± 1.0 (8)*

Table V. Seizure onset times following nerve agent exposure and treatment with various oxime doses[1]

[1] Guinea pigs received pyridostigmine bromide (0.026 mg/kg, i.m.) 30 min before receiving nerve agent (2.0 x LD50) challenge, followed 1 min later by atropine sulfate and oxime treatment. Seizure onset times (in minutes) are expressed as mean ± SEM (N).
*p<0.05 vs. GB and GF within oxime treatment.
ap<0.05 vs. DHAP and 2-PAM within agent group.
-- Not tested.

Time to seizure onset for VX was significantly different from both GB and GF ($p<0.05$) regardless of 2-PAM, MINA or DHAP treatment. Thus, the rank order for latencies to seizure onset for these nerve agents is VX > GB = GF, as was noted elsewhere [6, 7].

Seizure Termination.

In animals treated with atropine sulfate plus 2-PAM at one min after a nerve agent (at 2.0 x LD_{50}), the EEG seizure activity induced by GB, GF, or VX never abated, although at 24 hr the amplitude and frequency of spiking activity were significantly reduced in the survivors. Similar results were observed for MMB-4 treatment in animals exposed to 2.0 x LD_{50} GB. Figure 4 shows a typical EEG tracing of the ineffectiveness of MMB-4 to terminate GB-induced seizure activity.

In animals treated with MINA at doses that were sufficient to terminate EEG seizure activity, the average times to seizure termination from treatment (mean ± SEM) for a 2.0 x LD_{50} GB, GF, and VX challenge were 9.9 ± 2.8 (N=3), 19.7 ± 9.9 (N=7), and 82.6 ± 17.1 (N=6) min, respectively (Table VI). The time to seizure termination for VX was significantly different from either GB or GF ($p<0.05$). Figure 5 displays the GB-induced EEG seizure activities (seizure onset at 3.9 min) that were terminated by MINA (35 mg/kg) at 7.5 min after seizure onset. In DHAP-treated animals the average times to seizure termination from treatment (mean ± SEM) for a 2.0 x LD_{50} GB and VX challenge were 47.8 ± 12.7 (N=6) and 86.5 ± 19.9 (N=3) min, respectively (Table VI). DHAP was unable to terminate seizures in GF-exposed animals.

Body weight loss.

Animals that were treated with 2-PAM or MMB-

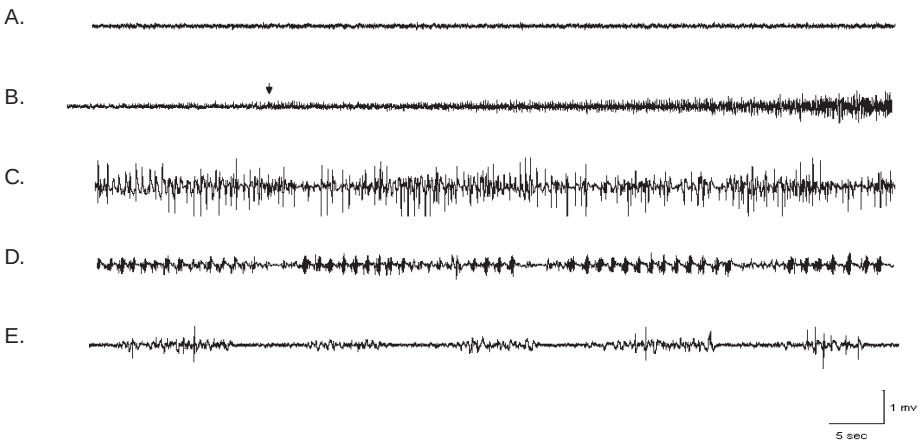


Figure 4. EEG Tracings of Guinea Pig Treated with MMB-4

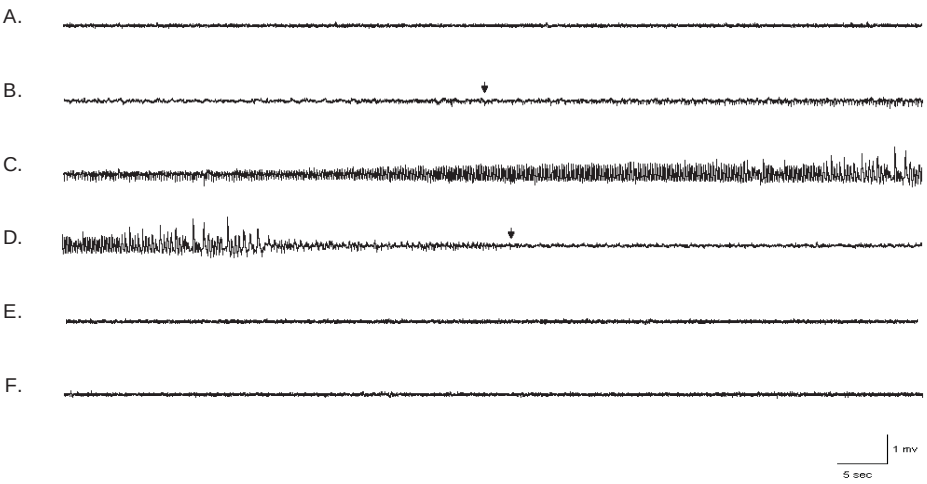


Figure 5. EEG Tracings of Guinea Pig Treated with MINA

Agent	MINA	DHAP
GB	9.9 ± 2.8 (3)	47.8 ± 12.7 (6)
GF	19.7 ± 9.9 (7)	No termination
VX	82.6 ± 17.1 (6)*	86.5 ± 19.9 (3)

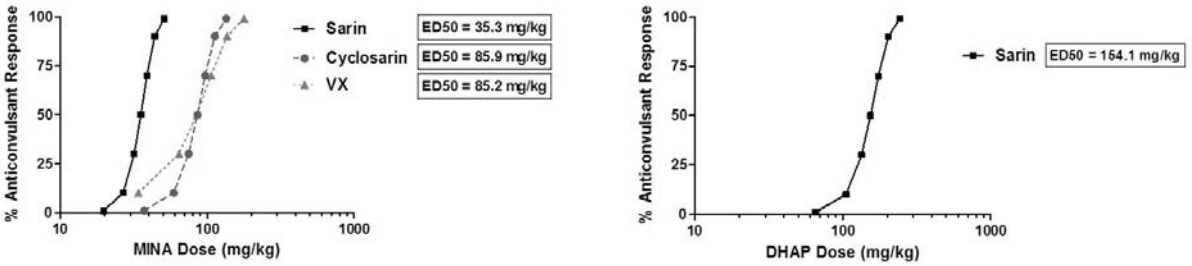
Table VI. Termination times for animals which experienced seizure activity[1]

[1]Guinea pigs received pyridostigmine bromide (0.026 mg/kg, i.m.) 30 min before receiving nerve agents (2.0 x LD50), followed 1 min later by atropine sulfate and oxime treatment. Times to seizure termination from time of oxime treatment (min.) are expressed as mean ± SEM (N).
* $p<0.05$ vs. both GB and GF

4 and survived to 24 hr experienced an average weight loss of 85 grams (about 30% of pre-exposure body weight). Those animals treated with MINA or DHAP and had seizures terminated experienced significantly less ($p < 0.001$) body weight loss over the 24 hr survival period (ranging from 16.6±2.7 [N=12] to 27.2±3.2 [N=10] grams) than did animals with seizures not terminated (ranging from 26.8±4.3[N=4] to 43.7±4.7 [N=3] grams). The 24 hr body weight loss in the latter group (i.e., seizures not terminated by MINA or DHAP treatment) was significantly less than in those animals treated with 2-PAM or MMB-4.

Anticonvulsant Efficacy.

The anticonvulsant responses of MINA and DHAP treatments at various doses against 2.0 x LD_{50} of GB, GF, and VX are shown in Table III,



A. MINA **B. DHAP**

Figure 6. Anticonvulsant effects of MINA and DHAP against sarin, cyclosarin, or VX

while Figures 6A and 6B display the anticonvulsant dose-response curves of MINA and DHAP, respectively, in our anticonvulsant test model. The anticonvulsant ED_{50} (with 95% confidence intervals) for MINA in the presence of atropine sulfate against 2.0 x LD_{50} of GB, GF, and VX was 35.3 (24.8 – 43.3), 85.9 (58.5 – 109.3), and 85.2 (50.0 – 116.4) mg/kg, i.m., respectively. The anticonvulsant ED_{50} for MINA against GB was published earlier [17]. Thus, the capability of MINA to stop or prevent seizure activity induced by these three nerve agents was in the order of GB > VX = GF (Figure 6A). The anticonvulsant ED_{50} for DHAP against GB was determined to be 154.1 (127.9 – 224.4) mg/kg, i.m. (Figure 6B). The anticonvulsant ED_{50} doses of DHAP against GF or VX (although 4 of 6 animals showed anticonvulsant response at 180 mg/kg) could not be determined (see Table IIIC for anticonvulsant response). Overall, the anticonvulsant efficacy for MINA was significantly more potent than that for DHAP under similar conditions.

Discussion

The toxic and lethal consequences of OP nerve agent intoxication are due to the irreversible inhibition of the critical cholinergic enzyme AChE which serves to hydrolyze and degrade the released cholinergic neurotransmitter ACh [1]. The currently used antidotes include an oxime reactivator (such as 2-PAM, obidoxime, or HI-6) to rescue the activity of nerve agent-inhibited AChE. However, these oximes have quaternary nitrogen structures and do not readily penetrate the BBB to reactivate brain AChE, limiting their therapeutic action to only peripheral AChE [22]. Thus, the nerve agent-induced central neurobehavioral adverse effects have to be mitigated in general by supplemental administration of an anticholinergic such as atropine sulfate and a benzodiazepine such as diazepam or midazolam [1-4, 28, 31]. These drugs themselves also cause psycho-pharmacological and sedative effects. It is our belief that the best nerve agent antidote is an AChE reactivator that can restore normal cholinergic neurotransmission both in the periphery and in the CNS after nerve agent exposure.

In the present study we investigated the AChE reactivating ability and anticonvulsant capacities of two tertiary CNS-active oximes, MINA and DHAP, and compared their effectiveness with two quaternary oximes, 2-PAM and MMB-4, in GB-, GF- and VX-exposed guinea pigs. In the reactivation study, animals were challenged with a 1.0 x LD_{50} dose of GB, GF, or VX followed 15 min later by treatment with saline (served as control) or a selected dose of an oxime [22, 29]. CNS and peripheral tissues and blood were collected at 60 min after nerve agent exposure for AChE activity determination. Both quaternary and tertiary oximes were able to reactivate nerve agent-inhibited AChE, but exhibited tissue specificity. Only the tertiary oximes reactivated inhibited AChE in the CNS. MINA displayed a broader efficacy against all 3 nerve agents, while DHAP showed only effective against GB-inhibited brain AChE. Both the quaternary and tertiary oximes reactivated nerve agent-inhibited AChE in the periphery, although the degree of AChE recovery exerted by the tertiary oximes was in general lower than that produced by the quaternary oximes. In the anticonvulsant experiment, a standard guinea pig study model was used with a 2.0 x LD_{50} dose of a nerve agent to induce 100% incidence of seizure activity [6, 28]. MINA and DHAP, but not the quaternary oximes, prevented or quickly terminated seizure activity, with MINA displaying a higher potency (effective against all 3 nerve agents) than DHAP (only effective against GB). At higher MINA or DHAP doses, many animals never developed EEG seizure activity and lost only a modest amount of body weight over the 24 hr survival period. Thus, an oxime that can reactivate brain AChE inhibited by a nerve agent displayed reduced CNS adverse outcomes.

In the AChE reactivation study, we chose a 145

μmol/kg (=25 mg/kg) dose of 2-PAM, which is equivalent to 3 autoinjectors of the Mark I nerve agent antidote kit for a 70 to 75 kg human. On the other hand, a 58 μmol/kg (=26 mg/kg) dose was used for MMB-4 based on a 3-autoinjector equivalent dose (500 mg per injector) of HI-6 [23], since MMB-4 is a bis-pyridinium compound similar to HI-6. We also used 145 μmol/kg as a starting dose for MINA and DHAP. MINA at 145 μmol/kg (=12.6 mg/kg) was not able to reactivate GB-, GF or VX-inhibited AChE in blood and peripheral tissues (data not shown). The AChE reactivation in blood and peripheral tissues was observed only at much higher doses of MINA: at 55.5 mg/kg and above against GB and VX, and at 35.0 mg/kg and above against GF. The reason for this is not clear, but could be due to the ability of MINA to penetrate and distribute into the CNS compartment. DHAP at the dose of 145 μmol/kg (=35.5 mg/kg) was able to reactivate GB-inhibited AChE in the blood (RBC and WB) and 2 peripheral tissues (diaphragm and heart). As the doses of DHAP increased to 50 mg/kg and above all 3 peripheral tissue AChE activities were reactivated. However, much higher doses of DHAP (70.8 mg/kg and above) were required to reactivate GF-inhibited AChE in the WB and heart tissue but with weaker reactivating capacity than MINA (12.0 % vs. 34.7 % AChE recovery). DHAP showed minimum capacity to reactivate VX-inhibited AChE in any peripheral tissue. Thus, the hypothesis put forward by Benschop et al. [19] that DHAP provided advantages over MINA due to its low toxicity, its relatively high reactivation efficiency of GB-inhibited AChE, and its PKa that is similar to that of 2-PAM could not be verified against other OP nerve agents.

It is unclear why MINA and DHAP reactivated GF-inhibited AChE in these studies. Reactivation by oximes of GF-inhibited AChE is, in general, more difficult than that of GB- and VX-inhibited AChE [22]. Some very potent oximes (relative to MINA and DHAP), such as 2-PAM and HI-6, are poor reactivators of GF-inhibited AChE [22]. On the other hand, 2-PAM (at 145 μmol/kg) and MMB-4 (at 58 μmol/kg) reactivated GB- and VX-inhibited AChE in blood and peripheral tissues quite well, with MMB-4 showing a higher reactivating capacity than that of 2-PAM against VX-inhibited AChE. MMB-4 also reactivated GF-inhibited AChE in the blood and peripheral tissues, while 2-PAM only produced minimum reactivation of GF-inhibited AChE in the WB.

As can be expected, 2-PAM and MMB-4 did not show any AChE reactivation in the CNS, since they do not readily penetrate the BBB because of their quaternary structure and limited lipid solubility. On the other hand, MINA and DHAP are tertiary structures and highly lipid soluble and, both significantly reactivated GB-inhibited AChE in most of the brain regions studied. At effective doses of MINA and DHAP, MINA reactivated GB-inhibited AChE activity in more brain regions than DHAP did. The differences in the regional specificity of these two tertiary oximes are not understood but may be due to their individual biodistribution profile in brain regions. Additionally, at higher doses the AChE reactivating capacity in the CNS was highly significant for both MINA and DHAP, with MINA being more potent than DHAP. The differences in potency between these 2 oximes may be due to the small and more compact structure of MINA, which allows it to enter the OP nerve agent-AChE binding site more easily. Similarly, the bulkier molecular structure of DHAP may explain why it could not reactivate GF- and VX-inhibited AChE in the CNS. The small and compact structure of MINA, thus, readily reactivated GB-, GF-, and VX-inhibited AChE to the same degree (24%, 35%, and 22% recovery, respectively) in the CNS.

Another noteworthy observation in the reactivation study was that, while MINA produced a dose-dependent increase in brain regional AChE reactivation from 55.5 to 139.3 mg/kg, the reactivation profile of DHAP reached a plateau at 70.8 mg/kg in several brain regions. From the 100 to 141.3 mg/kg doses of DHAP, there was no further increase in AChE activity. The reason for this phenomenon, produced by DHAP but not by MINA, in the CNS is not clear, but again may be due to the bulkier structure of DHAP. Even though the CNS AChE reactivation produced by DHAP was not quite dose dependent beyond 70.8 mg/kg, animals treated with increasingly higher doses of both MINA and DHAP clearly displayed less toxic signs of GB-induced cholinergic hyperactivity than were observed in those animals treated with 2-PAM or MMB-4. These observations were confirmed by the results of our anticonvulsant study.

In the anticonvulsant study, both MINA and DHAP produced anticonvulsant effects against GB, with MINA having greater potency than DHAP (ED_{50} = 35.3 mg/kg vs. 154.1 mg/kg). When MINA or DHAP was administered along with atropine sulfate in PB-pretreated guinea pigs, EEG seizure activity induced by 2.0 x LD_{50} of GB was prevented or quickly arrested. As the doses of MINA or DHAP increased, less of the animals exhibited EEG seizure activity. If seizure activity did occur, it was spontaneously terminated within minutes. Thus, increasing the doses of MINA or DHAP reduced seizure occurrence and increased the propensity for seizure termination. Additionally, increases in the dose of MINA or DHAP were observed to enhance survival and minimized 24

hr weight loss. These observations confirmed our notion that control of nerve agent-induced seizure activity is key to survival [6]. On the other hand, the quaternary oximes, 2-PAM and MMB-4, had no effect on GB-induced seizure activity and, while preventing lethality to some degree, did not quite protect against the GB-induced body weight loss.

In view of current pharmacological data, it was unfortunate that tertiary oximes were not pursued further in the 1950s because of the earlier reports that quaternary pyridinium oximes were several orders of magnitude better reactivators of phosphorylated AChE in human erythrocytes [15]. Both MINA and DHAP are lipid soluble and can readily penetrate the BBB [8, 19], as was confirmed with our reactivation study. The resulting CNS AChE reactivation has beneficial functional consequences as shown with the current anticonvulsant study. When MINA or DHAP was administered at high doses one min after 2.0 x LD_{50} of GB, many of the animals never displayed EEG seizure activity, or the bursting seizure activity was quickly terminated. The tertiary oximes had been shown to reactivate AChE within the CNS [8, 9]. Our present and earlier data not only supports these earlier findings, but further demonstrates that effective reactivation of AChE in the brain can increase survival and prevent seizure and possibly the subsequent neuropathology [17]. Askew [10, 11] showed in the late 1950s that when used alone or in combination with atropine sulfate, MINA markedly raised the LD_{50} doses of GB in mice, rats, guinea pigs, and rabbits. In our anticonvulsant study, we confirmed that both MINA and DHAP increased survival in guinea pigs. We also showed that both MINA and DHAP possessed anticonvulsant effectiveness against GB, with MINA being more potent in this respect. It has been our observation [5-7, 28, 31] that if nerve agent-induced seizure activity can be eliminated rapidly with an effective anticonvulsant treatment, there will be less brain pathology and a high probability of survival. Including MINA or DHAP in a therapeutic regimen for GB poisoning can be reasonably predicted to significantly increase survival and reduce brain pathology and associated behavioral abnormalities that would be seen in survivors.

Such beneficial antidotal effects can be extended to GF and VX exposures when MINA is administered soon after acute toxic exposure to these two nerve agents as well, although a slightly higher dose of MINA would be required. The broad-spectral effects of MINA therapy are, however, not observed with DHAP treatment. The reason for this can be explained by our findings that MINA reactivated brain AChE inhibited by GB, GF and VX, whereas DHAP reactivated only brain AChE inhibited by GB. This again supports the notion that CNS AChE reactivation has beneficial functional consequences. MINA showed a dose-dependent reactivation of AChE activity in both central and peripheral tissues against GB, GF, and VX. As the dose of MINA increased, more inhibited AChE was reactivated in more tissues. MINA was, however, a weaker reactivator of peripheral AChE than were the quaternary oximes [22]. These findings suggest that a combination treatment containing both a quaternary and a tertiary oxime would effectively reactivate nerve agent-inhibited AChE in the CNS and peripheral tissues.

In conclusion, this study clearly shows that when a CNS active oxime (MINA or DHAP) reactivated AChE in the brain, it reduced toxic signs and body weight loss, improved survival, and prevented or spontaneously terminated seizures following GB, GF, or VX intoxication. The current results support the notion that central AChE reactivation or preservation of CNS AChE activity following OP nerve agent intoxication is critical in the medical management of nerve agent intoxication [29, 32-34]. Thus, a tertiary oxime with high lipid solubility could be an excellent adjunct to current pretreatment and therapy regimens [3, 4] for the medical management of OP nerve agent intoxication.

Acknowledgements

The excellent technical team work of Cindy Acon-Chen, Katelyn Black, Jessica K. Chandler, Claire Eisner, James F. Irwin, Emylee C. McFarland, Amy J. Wegener, and Kristin M. Winter is acknowledged. This project was funded by the Defense Threat Reduction Agency – Joint Service and Technology Office, Medical Science and Technology Division. Portions of the work with the AChE reactivation experiment of MINA and 2-PAM and the anticonvulsant experiment of MINA and 2-PAM against GB have been published earlier [17, 29].

The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the United States Government. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. This research was supported by the Defense Threat Reduction Agency – Joint Science and Technology Office, Medical S&T Division.

References

1. Taylor P. 2001. Anticholinesterase agents. In: **Hardman JG, Limbird LE, Gilman AG (Eds.),** *Goodman and Gilman's The Pharmacological Basis of Therapeutics,* **10th edition, McGraw-Hill: New York, pp. 175-191.**

2. Wilson IB, Ginsburg A. 1955. Powerful reactivator of alkylphosphate-inhibited acetylcholinesterase. *Biochem. Biophys. Acta.* **18: 168-170.**

3. Moore DH, Clifford CB, Crawford IT, Cole GM, Baggett JM. 1995. Review of nerve agent inhibitors and reactivators of acetylcholinesterase. In: **Quinn DM, Balasubramanian AS, Doctor BP, Taylor P (Eds.),** *Enzymes of the Cholinesterase Family,* **1st edition, Plenum Press: New York, pp. 297-304.**

4. Aas P. 2003. Future considerations for the medical management of nerve-agent intoxication, Prehosp. *Disaster Med.* **18: 208-216.**

5. McDonough JH, Shih T-M. 1997. Neuropharmacological mechanisms of nerve agent-induced seizure and neuropathy. *Neurosci. Biobehav. Rev.* **13: 559-579.**

6. Shih T-M, Duniho SM, McDonough JH. 2003. Control of nerve agent-induced seizures is critical for neuroprotection and survival. *Toxicol. Appl. Pharm.* **188: 69-80.**

7. Shih T-M, Rowland TC, McDonough JH. 2007. Anticonvulsants for nerve agent-induced seizures: the influence of the therapeutic dose of atropine. *J. Pharmacol. Exp. Ther.* **320: 154-161.**

8. Cohen EM, Wiersinga H. 1960. Oximes in the treatment of nerve gas poisoning. *Acta Physiol. Pharmacol.* **9: 276–302.**

9. Rutland JP. 1958. The effect of some oximes in sarin poisoning. *Br. J. Pharmacol.* **13: 399-403.**

10. Askew BM. 1956. Oximes and hydroxamic acids as antidotes in anticholinesterase poisoning, *Brit. J. Pharmacol.* **11: 417-423.**

11. Askew BM. 1957. Oximes and atropine in sarin poisoning, *Brit. J. Pharmacol.,* **12: 340-343.**

12. Dultz L, Epstein MA, Freeman G, Gray EH, Weil WB. 1957. Studies on a group of oximes as therapeutic compounds in sarin poisoning. *J Pharmacol Exp Ther.* **119: 522-531.**

13. Myers DK. 1959. Mechanism of the prophylactic action of diacetylmonoxime against sarin poisoning, *Biochem. Biophys. Acta.* **34: 555-557.**

14. Wills JH. 1959. Recent studies of organic phosphate poisoning. *Fed. Proc.* **18: 1020-1025.**

15. Hobbiger F. 1963. Reactivation of phosphorylated acetylcholinesterase, In: Koelle GB (Ed.), Cholinesterases and Anticholinesterase Agents, *Handbuch der Experimentellen Pharmakologie,* **Springer-Verlag, Berlin, pp. 921-988.**

16. Shih T-M, Skovira JW, O'Donnell JC, McDonough JH. 2009. Central cholinesterase reactivation by oximes improves survival and terminates seizures following nerve agent intoxication. *Adv. Studies Biol.,* **1: 155-196.**

17. Shih T-M, Maxwell DM, Koplovitz I, Kan RK, McDonough JH. 2010. Reactivation of acetylcholinesterase activity and its therapeutic benefits in nerve agent intoxication. In: **Weissman BA, Raveh L (Eds.),** *The Neurochemical Consequences of Organophosphate Poisoning in the CNS,* **Transworld Research Network: Kerala, India, pp. 111-133.**

18. Shih T-M, Skovira JW, O'Donnell, JC and McDonough JH. 2010. Treatment with tertiary oximes prevents seizures and improves survival following sarin intoxication. *J. Mol. Neurosci.* **40: 63-9.**

19. Benschop HP, De Jong LRA, Vink JAJ, et al. 1976. The prophylactic value of oximes against organophosphate poisoning. In: **SIPRI,** *Medical Protection against Chemical-Warfare Agents,* **Almqvist & Wiksell International: Stockholm, pp. 120-33.**

20. Singh H, Moorad-Doctor D, Ratcliffe RH, Wachtel K, Castillo A, Garcia GE. 2007. A rapid cation-exchange HPLC method for detection and quantification of pyridinium oximes in plasma and tissue. *J. Analy. Toxicol.* **31: 69-74.**

21. Saxena A, Luo C, Chilukuri N, Maxwell DM, Doctor BP. 2008. Novel approaches to medical protection against chemical warfare nerve agents. In: **Romano JA, Lukey JA, Salem H (Eds.),** *Chemical Warfare Agents: Chemistry, Pharmacology, Toxicology and Therapeutics,* **Second edition, CRC Press, Boca Raton, pp. 145-173.**

22. Shih T-M, Skovira JW, O'Donnell JC, McDonough JH. 2009. Evaluation of nine oximes on in vivo reactivation of blood, brain, and tissue cholinesterase activity inhibited by organophosphorus nerve agents at lethal dose. *Toxicol. Mech. Methods.* **19: 386-400.**

23. Clair P, Wiberg K, Granelli I, Carlsson BI, Blanchet G. 2000. Stability study of a new antidote drug combination (Atropine-HI-6-prodiazepam) for treatment of organophosphate poisoning, *Eur. J. Pharm. Sci.* **9: 259–263.**

24. Vallejo-Freire AA. 1951. A simple technique for repeated collection of blood samples from guinea pigs. *Science.* **114: 524-525.**

25. Shih T-M, Kan RK, McDonough JH. 2005. *In vivo cholinesterase inhibitory specificity of organophosphorus nerve agents. Chem. Biol. Interact.* **157-158: 293-303.**

26. Shih T-M, Romano JA. 1988. Effects of choline on soman-induced analgesia and toxicity. *Neurotoxicol. Teratol.* **10: 287-294.**

27. Ellman GL, Courtney KD, Andres V, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7:88–95.**

28. McDonough JH, Zoeffel LD, McMonagle J, Copeland TL, Smith CD, Shih T-M. 2000. Anticonvulsant treatment of nerve agent seizures: anticholinergics versus diazepam in soman-intoxicated guinea pigs. *Epilepsy Res.* **38: 1-14.**

29. Skovira JW, O'Donnell JC, Koplovitz I, Kan RK, McDonough JH, Shih T-M. 2010. Reactivation of brain acetylcholinesterase by monoisonitrosoacetone increases the therapeutic efficacy against nerve agents in guinea pigs. *Chemico-Biological Interactions* **187: 318–324.**

30. Bliss CI. 1952. The statistics of bioassay with special reference to the vitamins. **In:** *Vitamin Methods,* **Academic Press: New York.**

31. McDonough JH, McMonagle J, Copeland T, Zoeffel D, Shih T-M. 1999. Comparative evaluation of benzodiazepines for control of soman-induced seizures. *Arch. Toxicol.* **73: 173-478.**

32. Fosbraey P, Wetherell JR, French MC. 1992. Effect of acute physostigmine-hyoscine pretreatment on the neurochemical changes produced by soman in the guinea pig. *Neurochem. Int.* **18: 265–272.**

33. Wetherell JR. 1994. Continuous administrations of low dose rates of physostigmine and hyoscine to guinea pigs prevents the toxicity and reduces the incapacitation produced by soman poisoning. *J. Pharm. Pharmacol.* **71: 1023–1027.**

34. Wetherell JR, Hall T, Passingham S. 2002. Physostigmine and hyoscine improves protection against the lethal and incapacitating effects of nerve agent poisoning in the guinea pig. *Neurotoxicology.* **23: 341–349.**